III. Experimental and clinical immunology

SUPPRESSOR T LYMPHOCYTES IN PREGNANCY

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The mechanisms leading to maternal tolerance of the fetal allograft during the period of gestation are not yet completely elucidated. It is known that pregnant subjects develop an immune response directed against various antigens expressed on the fetus and including major and minor histocompatibility antigens of paternal origin (7). The humoral limb of this maternal immune response is well documented, whereas its cellular counterpart is much less known. The difficulty to demonstrate maternal cell-mediated response to her fetus may reflect a state of partial tolerance affecting T lymphocytes, the main effectors of graft rejection, but sparing the B lymphocyte system, capable of producing «blocking» of protective antibodies enhancing the survival of the fetal graft (8, 18).

In the present study we tested the in vitro reactivity of maternal T lymphocytes to paternal antigens by measuring both their proliferative and cytotoxic responses to paternal and cord blood cells. The results failed to demonstrate that during normal pregnancy there is an active in vivo sensitization of circulating T lymphocytes to paternal antigens. The data further suggest that these negative findings might be related to the presence among maternal circulating lymphocytes of suppressor T cells, characterized by their reactivity with the OKT_8 monoclonal antibody and their membrane receptors for the Fc fragment of IgG (T_γ cells).

Material and methods

Donors of lymphocytes: Peripheral blood lymphocytes (PBL) were obtained from three groups of healthy individuals: last-trimester pregnant women with a normal pregnancy, their husbands and unrelated blood donors. The age of the donors was comprised between 18 and 37 years; the parity of the pregnant participants is indicated in the results. The cord blood samples were all obtained after vaginal deliveries of healthy newborns.

Isolation of mononuclear cells: Cord blood was drawn into sterile tubes containing heparin before the expulsion of placenta. Red cells were allowed to sediment for 45 min in the presence of 20 % (v/v) dextran (MW 200,000:5 % in saline); the leukocyte-rich supernatant was then mixed with 2 volumes of Hank's (Flow Lab) before being centrifuged on Ficoll-Hypaque. Peripheral blood lymphocytes (PBL) were obtained from heparinized blood by Ficoll-Hypaque centrifugation. Cells were washed 4 times in Hank's, enumerated and resuspended in RPMI 1640 (Flow Lab) supplemented with 1 μg/ml gentamycin, 5 mM or L- gluta-

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mine and 20 % heat inactivated pooled human serum. Before culture, mononuclear cells were first depleted in adherent cells according to the method of Mosier and Coppleson (12) as previously described (21).

Preparation of T cell-rich lymphocyte population: These were obtained by rosetting mononuclear cells with sheep red blood cells pretreated with aminoethylthiouronium hydrobromide (AET, Aldrich Chemical Co.) as described (9) and by centrifuging the mixture over a Ficoll-Hypaque gradient; T cells were separated from sheep red blood cells by ammonium chloride lysis.

T lymphocyte with receptors for IgG were isolated as described by Moretta et al. by rosetting T cell-rich preparations with ox red blood cells coated with rabbit IgG anti-ox antibodies (11).

Complement-dependent lysis of T_8^+ cells: Monoclonal antibody to the T cell antigen T_8 (OKT_8) expressed on human suppressor and cytotoxic cells (15) was obtained from Ortho Diagnostics (Raritan, U. S. A.). Lysis of T_8^+ cells was performed by incubating $3—5 \times 10^6$ T lymphocytes suspended in 0.5 ml of Hank's solution (HBSS) supplemented with OKT_8 at the final dilution of 1/250; after 1 hr at room temperature 150 µl of newborn rabbit serum was added and the mixture was incubated for an additional 50 min at 37 °C. Cells were then washed three times in Hank's before being resuspended in complete culture medium. Control cells were incubated with newborn rabbit serum alone. Viability of the T_8-depleted lymphocytes was greater than 95 % as estimated by trypan blue test; the content of these cellular suspensions in T_8^+ cells was less than 2 % as estimated by membrane fluorescence.

Mixed lymphocyte reaction (MLR) was performed by coculturing 10^5 mitomycin-treated stimulating cells with 10^5 responding lymphocytes from a different donor in 0.2 ml culture medium, for 6 days at 37 °C in 5 % CO_2 and air. Eighteen hours before harvesting with a Mash II cell harvester (Microbiological Associates) 1 Ci of ^{3}H-thymidine (Amersham; methyltritiated thymidine; 5 Ci/mM) was added to each well. Stimulating lymphocytes were inactivated by a 30 min incubation at 37 °C in the presence of 50 g/ml mitomycin C (Sigma Lab.)., followed by 3 washes in HBSS containing 10 % human serum.

Lymphocyte mediated cytotoxicity (LMC). At the end of day 6 mixed lymphocyte culture cells from 6 replicate wells were pooled, washed twice enumerated and resuspended in RPMI supplemented with 4 mM L-glutamine, 30 mM HEPES and 10 % pooled human serum. Target cells were first maintained for 2 days at 37 °C in tissue culture medium at the concentration of $1—2 \times 10^6$/ml, they were then stimulated with 1 g/ml PHA (PHA-P, Wellcome) for 72 hrs prior to labelling with ^{51}Cr. This was performed by incubating 1×10^6 lymphocytes in 0.9 ml culture medium with 0.1 ml $Na_2{}^{51}CrO_4$ (1000 Ci/ml, Amersham Lab.) for 18 hrs at 37 °C. Labelled cells were then washed 3 times in tissue culture medium and further incubated for 60 min at 37 °C; after a final wash target cells were suspended at the concentration of 8×10^5 cells/ml. ^{51}Chromium release assay was performed in V shaped microtiter plates by mixing 100 l target cells with 100 l effector cells at various concentrations; the mixture was incubated for 6 hrs at 37 °C, the plates were centrifuged and 100 l of supernatant was counted in a gamma counter. Maximum release was measured in supernatants of cells treated with HCl, spontaneous ^{51}Cr release was determined in supernatants of wells containing target cells only. Spontaneous release was comprised between 5—20 %

of the maximum release. Results of quadriplicate determinations are expressed as specific release calculated according to the formula:

$$\% \text{ specific cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous release cpm}}{\text{total release cpm} - \text{spontaneoue release cpm}} \times 100$$

Statistical analysis was performed by paired Student test or by two way variance analysis with replicates.

Results and discussion

In the first series of experiments, PBL freshly isolated from pregnant women during the last trimester of gestation, were tested for their spontaneous cytotoxic activity against their husband's lymphocytes. The latter were first preactivated in a 72 hr culture in the presence of PHA prior to their labelling with ^{51}Cr. Labelled target cells were then incubated with maternal PBL for 6 hr and 18 hr at 37 °C. No specific chromium release could be detected in 11 consecutive experiments performed at effector/target cells ratios of 10 and 40; (data not shown). Seven of the eleven cases were multiparous women, 4 of whom had more than 3 children. Similar negative results were obtained by testing directly the cytotoxic activity of maternal PBL collected shortly after delivery and co-cultured for 6 hrs with fresh ^{51}Cr labelled neonatal lymphocytes. These negative findings have been repeatedly made in 9 experiments including 4 cases of multiparous women. The results clearly indicated the absence of in vivo activated maternal cytotoxic T cells specific to paternal antigens. In the next series of assays we compared the responses of paternal and maternal PBL to third party mitomycin-treated cells. As shown in table 1, the two groups of responding cells displayed comparable

Table 1

Influece of pregnancy on lymphocyte response in MLR and IMC against lymphocytes from husband and unrelated donors

Experiment number	Cells in culture								
	m — Fm			M + Fm			F + Dm		
	MLR	LMC		MLR	LMC		MLR	LMC	
		E/T 10	E/T 40		E/T 10	E/T 40		E/T 10	E/T 40
1	101.4±10.9	68	88	90.1±11.7	55	72	73.3±11	7	15
2	46.5± 2	25	35	37.9±3.7	32	45	85.3±7.3	37	43
3	140±2.8	10	20	161.4±15.8	27	45	105.4±17.3	25	35
4	58.1±6.7	42	58	12.2±2.2	13	22	25.6±5.4	12	48
5	96.8±6.3	17	30	160.6±19	12	20	170±6.4	16	23
Mean ± S.E.M.	88.4±16.7	32.4± 10.4	46.2± 12.1	92.2±7.8	27.8± 7.8	40.8± 9.4	92.1±13.6	19.4± 5.3	32.8± 6.1

proliferative and cytotoxic responses to the same unrelated allogeneic stimulating cells. The results thus indicated that pregnancy does not interfere with the ability of PBL to mount an in vitro cytotoxic response to an allogeneic stimulus.

In the same series of experiments we also observed that the maternal response to paternal alloantigens was not different from paternal lymphocytes to maternal antigens. These results do not suggest that maternal T cells have been primed to fetal antigens of paternal origin nor that they are tolerant to the same antigens. This view was further supported by similar experiments comparing paternal and maternal PBL responses to cord blood cells. In these assays PBL were isolated from the two parents at the time of delivery and were then divided into three aliquots: one was mixed in vitro with mitomycin-treated cord blood cells; another was treated with mitomycin and used as stimulant; the last aliquot was incubated in tissue culture medium alone for 2 days before being stimulated with PHA for another 3 days and then labelled with ^{51}Cr. The results summarized in table 2 showed that maternal and paternal lymphocytes displayed similar responses to cord blood cells. As expected, adult cells were more potent stimulators than neonatal cells which share half of their histocompatibility antigens with each of the parent. Note also that, as in the previous experiments (table 1) maternal and paternal lymphocytes were equally reacting to each other.

Table 2

Maternal and parental lymphocyte response in 6 day MLR and IMC against their child blood cells

MLR	M + Cm		M + Fm		F + Mm		F + Cm	
	43±10. p<0.01		66±15		92±27 p<0.01		54∓23	
	E/T 50	E/T 12.5	E/T 50	E/T 12.5	E/T 50	E/T 12.5	E/T 50	E/T 12.5
LMC	48.4±14	16.2±10	38.6±21	14.6±15	40±14	13±6	45±6	15.2±7

Since it is known that primed T cells exhibit an earlier peak response in mixed lymphocyte reaction (MLR) than unprimed T cells (19) our failure to demonstrate a sensitization of maternal lymphocytes to paternal antigens might be due to an inappropriate duration of the MRL. This possibility was tested in kinetic experiments comparing MLR of 3, 4, 5 and 6 days duration. Surprisingly, we observed that in 3 day mixed lymphocyte cultures, maternal lymphocytes were significantly less reactive to paternal than to unrelated mononuclear cells (table 3). In order to further analyze these findings, 3 day MLR were carried out in which purified T lymphocytes depleted or not of OKT_8^+ or T_γ cells were used as responding cells. The results of 21 experiments, illustrated in figures 1 and 2, may be summarized as follows:

1. Maternal T cells had a significantly lower response to paternal than to unrelated cells;
2. Maternal T cells depleted of T (fig. 1a) or OKT_8 cells (fig. 1 b) had a similar response to paternal and unrelated cells;
3. Both maternal and paternal T cells depleted of T_8 or T cells had higher responses than unfractionated T cells.

The above results were not influenced significantly by the parity of the female donors as shown by the comparison of the results from 14 multiparous to 7 primiparous individuals.

Table 3

MLR 3 day cultures

	M	S. E. M.	
M-Fm	7.896*	1.631	$p<0,02$
M-Mm	10.107	1.836	NS
F-Dm	12.781	1.957	

* CPMx10^3

M±S. E. M. of 15 experiments
M — maternal lymphocytes
F — paternal lymphocytes
Fm — paternal mitomycin-treated cells
Mm — maternal mitomycin-treated cells
Dm — third party mitomycin-treated cells

The data suggest not only that both T and OKT_8 cells had an inhibitory effect on the T lymphocyte proliferation in MLR but also that antigen-specific suppressor cells were present in the OKT_8 and T fractions of maternal lymphocytes. Indeed, after their removal, maternal T lymphocytes no longer showed a selectively reduced response to paternal antigens; even more in some experiments the T_γ or OKT_8 depleted T lymphocytes responded better to paternal than to unrelated alloantigens. In order to further investigate the latter observations, 6 day MLR have been carried out employing T and OKT_8 depleted T cells as responder cells. In 8 consecutive experiments we were not able to show a differential response of the maternal T cell subpopulations to paternal or third party stimulating cells (not shown).

The present studies failed to demonstrate the presence of activated or immunized cytotoxic T lymphocytes specific for paternal antigens in the peripheral blood of pregnant women. Indeed, freshly isolated PBL from primiparous and multiparous individuals had no spontaneous cytotoxic activity against neither PHA-activated paternal lymphoblasts nor unstimulated fresh cord blood cells. The data are contrasting with the observations by Rocklin (17) showing that lymphocytes from pregnant women release Migration Inhibiting factor (MIF) upon exposure in short term cultures to paternal or cord blood cells but not to third party cells. These contrasting results are nevertheless not in opposition since it is clearly established that the lymphocytes producing MIF and the cytotoxic cells belong to two different subpopulations (14). By using conventional 6 day MLR and CML assays we were not able to detect any significant difference between the responses in each test of maternal, paternal or third party cells to cord blood or adult stimulating and target cells. Thus, maternal PBL displayed the same reactivity in MLR and CML assays against alloantigens of paternal or unrelated origin and there was no difference between the responses of paternal or maternal T lymphocytes to their child cord blood cells. These results are in agreement with previous findings by others (2, 16) mainly obtained in MLR assays. It was important to test the maternal lymphocyte response, not only in MLR but also in CML, indeed, it has been shown in some experimental models of allograft tolerance that MLR responses may be normal whereas the ability to generate cytotoxic cells is suppressed (1, 23). The present findings that pregnancy does not alter the lymphocyte capability to develop in vitro normal MLR and cytotoxic responses to allogeneic cells are in keeping with several recent reports showing

only discrete modification of the lymphocyte function of pregnant women (21, 22). For example, the lack of maternal T cell sensitization to paternal antigens cannot be accounted for by the small modification of the proportions of helper or

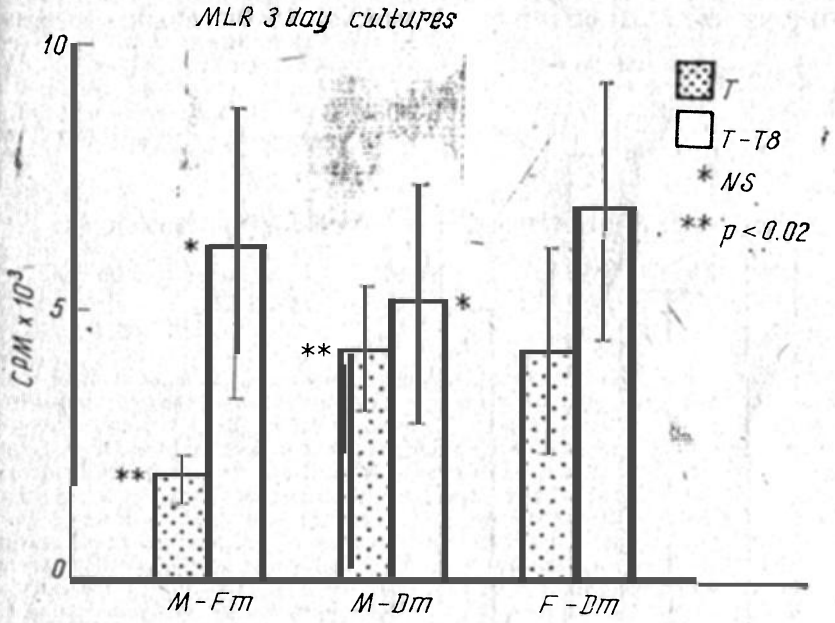

Fig. 1. Comparison of the responses of T lymphocytes, and Tγ-depleted T lymphocytes in a 3 day MLR. Mean values ± SEM of 12 consecutive experiments are shown

suppressor-cytotoxic lymphocytes observed during pregnancy (22). In the present experiments mitomycin-treated cord blood cells were less efficient stimulants of the parental lymhocytes than were paternal cells for maternal lymphocytes or vice versa. This is explained by the fact that neonatal cells share half of their histocompatibility antigens with each parent whereas it is likely that the parent does not have one of these antigens in common. These results can thus be explained without taking into account a possible suppressive action of the neonatal cells or the response of adult lymphocytes (13). In kinetic experiments we noted that after 3 day culture the proliferative response of maternal PBL was selectively depressed against paternal cells and that this impaired response was corrected by the removal of T or OKT_8 cells. One possible interpretation is that antigen-specific suppressor T cells, expressing both Fc IgG receptors and T_8 antigen, were inhibiting the maternal T lymphocyte response to the paternal antigens. Although this view needs to be confirmed in co-culture experiments with purified T, T_8 cells, it finds some support in the literature. First, this possibility is in keeping with two reports showing the presence of antigen-specific suppressor cells in pregnant women (6, 10) and second, the existence of suppressor cells has been well documented in experimental animals (4, 20). Chaouat and Voisin have characterized in the spleen of multiparous mice, a subpopulation of Ly_{12} T cells capable of selectively suppressing the induction of cytotoxic T cells specific to paternal antigens (3, 4). Clark et al. (5) have documented the early appearance

of suppressor lymphocytes in the decidua and in the local lymph nodes draining the uterus of pregnant mice. These suppressor cells which are non-T and non-B lymphocytes but display natural killer activity seem to play an essential role already at the stage of implantation of the blastocyst in the uterine mucosa

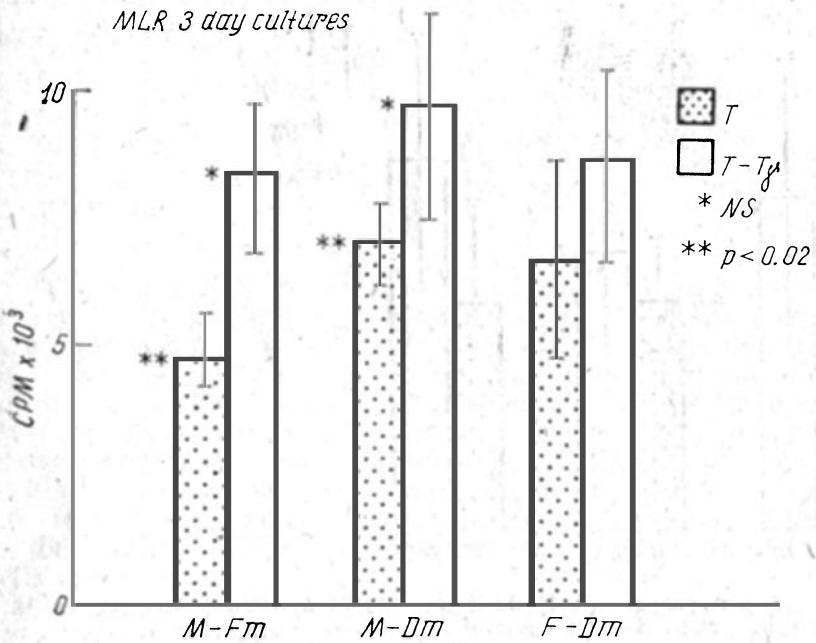

Fig 2 Comparison of the responses of T lymphocytes, and T_g-depleted T lymphocytes in a 3 day MLR. Mean values±SEM of 9 consecutive experiments are shown

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СУПРЕССОРНЫЕ Т-ЛИМФОЦИТЫ ВО ВРЕМЯ БЕРЕМЕННОСТИ

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РЕЗЮМЕ

Исследована лимфоцитарная реактивность к отцовским или неонатальным клеткам в смешаной лимфоцитарной культуре и в пробах для определения клеточно-опосредствованной цитотоксичности у здоровых рожающих в первый раз женщин и у много раз родивших женщин. Исследование проводится в конце беременности или непосредственно после рождения. Свежие изолированные лимфоциты матери не обладают спонтанной цитотоксической активностью к ФГА-активированным неонатальным или отцовским лимфоцитам. В конвенциональных 6-дневных смешаных лимфоцитарных культурах при опытах для установления цитотоксичности материнские лимфоциты ответили почти так же, как и отцовские и на лимфоциты третьей группы при стимулировании неонатальными лимфоцитами, отцовскими, или лимфоцитами третьей группы. В отличие от этого 3-дневные смешаные лимфоцитарные культуры материнских клеток дали селективно более слабый ответ на отцовские антигены (выраженные или на клетках крови, взятой из пуповины или же на отцовских лимфоцитах) по сравнению с несвязанными алоантигенами. Удаление Т-лимфоцитов с помощью рецепторов LgG (T_γ), или Т-лимфоцитов, реагирующих на ОКТ$_8$-моноклональные антитела, корригировало подавление ответа материнских клеток в этих 3-дневных культурах. Сделано предположение, что циркулирующие материнские лимфоциты содержат антигенно-специфические супрессорные клетки, характеризующиеся наличием мембранных рецепторов LgG и T_8-антигенов.